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STUDIES OF THE MITOTIC FIGURE

I. Chaetopterus: Central Body Structure at Metaphase, First Cleavage, After Picro-Acetic Fixation

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I. Purpose of the Study

Several years ago, in studies of central bodies in *Echinarachnius* eggs, the writer reached the conclusion that in the cytasters, spermasters, and first-cleavage figures of this egg the central body is a coagulation product of the area of focalized rays and spindle fibers, having no existence as an individualized body in the living cell (Fry, 1928, etc.).

To ascertain whether this situation exists in other forms, a number of studies have now been made of various cell types, including spermatocytes, oöcytes, recently fertilized eggs, early and late blastomeres and somatic cells, in various organisms, ranging from coelenterates to vertebrates. The central bodies in these cells exhibit such a wide diversity in behavior that the writer has made a provisional classification of centrioles and centriole-like bodies, which is discussed later (p. 181). Each class is identified by some phase of behavior not shared by the others. In the case of each type of cell investigated the purpose was to find out to which class the centriole belongs. After all of these results have been reported, the interrelations of the different classes will be discussed in a later paper.

The material of the present study, which is the first of the group just mentioned, is the egg of *Chaetopterus pergamentaceus*. Mead (1898) described typical centrioles in this egg which maintain genetic continuity from one cell cycle to the next. Wilson (1930) recently re-examined the original preparations of that work and confirmed Mead's observations. Some years ago the writer attempted to duplicate Mead's findings, using Boveri's picro-acetic reagent. Mead states that of the fixatives he employed this gave the best results. In the majority of the cells the writer studied, however, the astral centers were disrupted. A

group of experiments was therefore carried out, using this same reagent, for the purpose of ascertaining what phase of technique was responsible for Mead's demonstration of centrioles on the one hand, and the writer's inability to repeat the work on the other. Study was confined to metaphase asters of first-cleavage figures, because in general central bodies are most readily demonstrated about the time of metaphase, even if they are not present at earlier or later phases; and the mitotic figure of first cleavage has the advantage of its unusually large size.[1]

II. Methods

Treatment of Living Eggs

Prior to fertilization, the eggs were divided into three lots,[2] and fertilized at five-minute intervals, e.g., 9:55, 10:00, and 10:05. They were later mixed and the average time, e.g., 10:00, was regarded as the time of fertilization. Hence when eggs are fixed for the purpose of securing them at metaphase, which occurs in about 52 minutes at 21° C., there is a "spread" of stages from early prophase to late anaphase, and many eggs are in metaphase even if development has been retarded or accelerated in any one egg-set.

After fertilization the eggs were placed in a 1000 cc. crystallizing dish filled with sea water and kept covered to avoid evaporation and consequent modification of osmotic pressure. The water was changed every ten minutes and the eggs were gently stirred every two or three minutes. This avoids possible effects of overcrowding, keeps the oxygen supply normal, and removes metabolic wastes. The dish was immersed in a water bath and kept at a temperature of 21° ± .5. About five minutes before fixation the eggs were transferred to small Stender dishes. Just prior to fixation the water was poured off from each dish, leaving at the bottom a dense mass of eggs, together with a minimum amount of sea water, which necessarily accompanies the eggs. All egg-sets used showed more than 95 per cent first cleavage.

[1] Appreciation is expressed to Miss Sara J. Reynolds for her aid as research assistant in the work.

[2] The parapodia were cut up in a small amount of sea water, then placed on wet cheesecloth drawn taut over an empty bowl, and water was gently squirted through the mass until all eggs were released. Thereafter the water was changed every five minutes for the first half hour, during which maturation reaches metaphase of the first division. The eggs of two or more females were used in each egg-set, and they were fertilized about half an hour after they were secured. The sperm suspension was prepared by adding one drop of thick seminal fluid to about 10 cc. of sea water. Several drops of this were added to the eggs in 250 cc. of water. The exact dilution of the sperm suspension is unimportant, since polyspermy rarely occurs.

Fixation and Slide-making

The picro-acetic reagent employed in the experiments was made according to Boveri's formula (1887, p. 11): 99 parts of a saturated solution of picric acid which has been diluted with two volumes of water, and one part of glacial acetic acid. In experiments where this formula was modified the details are given in each case. Unless otherwise stated, eggs were run up in the usual manner, sectioned at a thickness of 5 μ, and stained in Heidenhain's hæmatoxylin.

Making Observations

It is well known that the two asters of the first-cleavage figure in *Chaetopterus* eggs differ in size (Fig. 1). In this paper only the larger aster of each figure was studied, in order to exclude possible variations due to differences in astral size.

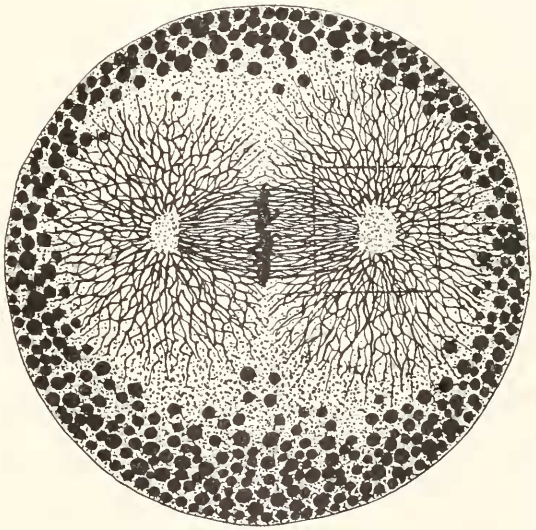


FIG. 1. The metaphase first-cleavage figure in *Chaetopterus* eggs after using Boveri's picro-actetic fixation.

The size difference between the two asters is apparent. The area outlined by the dotted lines is that part of the figure illustrated in the charts. There is here shown that type of structure occurring most frequently after using Boveri's picro-acetic fixation.

Metaphase is here regarded as the time when chromosomes are aligned in a flat plate. Eggs in late prophase, when the chromosomes are in a broad irregular group, and in early anaphase, when they are just beginning to separate, were excluded.

Only the mid-section of the large aster was studied. The sections at the left and right were always examined in order to be sure which was the mid-section. If any section was missing that egg was discarded.

A 4 mm. high dry objective was used in making a list of readings. When several types of central bodies occur on the same slide, as is usually the case, one may stand out more distinctly than the others, especially when such an objective is used. To make sure that a random sample of the egg population of each slide was secured, and to eliminate unconscious selection of any class, the slide was searched systematically by the use of a mechanical stage, and every metaphase figure was listed until the desired number was obtained. These cells were then studied under critical lighting conditions at magnifications of 600 or 900 ×, using an objective having a numerical aperture of 1.4 and a similarly corrected condensor.

Illustrations

In this study, as well as in those to be reported later, it is as important to illustrate the details of ray structure as of central bodies, since the major conclusion of the work is that the structure of the one is closely related to that of the other. Four types of illustration were tried: photographs made with white light, photographs made with ultra-violet light, wash drawings, and ink drawings.

Photographs made with white light show adequately the structure of the central body, but, owing to the nature of the photographic process,[3] they fail completely to show the finer details of ray structure so obvious to the eye.

Through the cooperation of Dr. F. F. Lucas, of the Bell Telephone Laboratories, ultra-violet photographs were made of the asters of *Chaetopterus* and several other species. They will be reported in a later paper, since they will have more significance if discussed after the regular cytological studies have been completed. Such photographs show delicate detail with maximum clarity; but until the technique has been simplified and made less expensive, they cannot have wide-spread use in cytological studies. Hence they are not employed in this group of papers as the regular mode of illustration.

[3] If, for example, a photograph is made of a series of alternating dark grey and light grey lines which have a relative intensity of 10 to 1, the relative intensity of the images of those lines on the negative is 2 to 1. This phenomenon has no practical consequence when photographing such objects as chromosomes or typical centrioles which are in distinct contrast to the surrounding material. But wherever there are only slight differences in intensity, and where the pattern is a delicate one, as in the case of ray and inter-ray materials, a photograph is capable of showing only a vague and unsatisfactory image of the actual structure.

Drawings of any kind, whether wash or ink, have the disadvantage of possibly showing unconscious over-emphasis of certain points or inadvertent omission of others; but they can show delicate details not reproduceable by white light photography, and they have the distinct advantage of showing what the eye sees at various levels of the preparation. In most cytological papers drawings of both astral rays and spindle fibers are frankly schematized, since the exact and literal delineation of the complicated ray pattern presents practically insurmountable obstacles—obstacles not associated with illustrations of most cell components. In many cases, e.g., Mead's wash drawings of *Chaetopterus* asters (1898), the metaphase rays are shown as straight, whereas they are actually more or less undulating. If ink drawings are used, as is often done, where rays are usually shown as black lines and inter-ray materials are not shown at all, the illustration has a degree of contrast much greater than that in the preparation. Furthermore, completely homogeneous areas, such as some large centrosomes, can be shown only by closely-placed dots, which give a granular effect unlike that of the original structure.

Wash drawings are unquestionably more pleasing aesthetically than ink drawings, for the use of several tones of grey and black results in an illustration having a general appearance more like that of the original preparation than is possible when only black is used. But both types can be either accurate or inaccurate in delineation of structure; and when such complex detail as the exact configuration of astral rays is illustrated both wash and ink drawings are inevitably more or less schematized. To show differences in the coarseness and the shape of rays in various astral types, ink drawings are capable of giving as adequate a picture of structural variations as are those made with the wash technique.

Ink drawings have been selected as the mode of illustration in this group of papers, because they can be embodied in charts, reproduceable with zinc plates, which are impracticable when wash drawings are used. Thus variations in astral structure can be related in a graph-like manner to the modifications of various experimental conditions, and there is apparent at a glance the relative frequency of each type under each condition.

Only the central region of the aster is shown in each drawing—an area indicated by the dotted lines in Fig. 1, page 151. If entire cells were shown at the magnification used, 1000 ×, they would occupy too much space to be included in charts.

The drawing of each of the twelve astral types occurring in this study was made from a specific cell, chosen because it was typical of

the class. Each drawing, therefore, not only delineates a single "best" cell, but it also represents the type.

Terminology

Following Wilson's usage, (1928, pp. 30 and 672–675) the term *centriole* is used to indicate a minute darkly-stained granule-like body; the term *centrosome* refers to a larger, more variable structure that often surrounds the centriole or may exist by itself. A *centrosphere* is a large vacuolar area at the astral center. The term *central body* is a general one; it may apply to any or all of these structures, and hence includes all configurations from a minute granule to a large empty area.

Mead uses these terms with different meanings: what is here termed a *centriole* he calls a *centrosome,* and what is here termed a *centrosome* he calls a *centrosphere.*

III. Experiments

Group A. Technique Standard: Boveri's Picro-Acetic Fixation and Regular Slide-Making Procedure

Experiment 1. The Relation Between Central Body Structure and Ray Structure

In this experiment the 243 eggs studied were selected at random from slides of various egg-sets which had been fixed, sectioned, and stained under supposedly similar optimum conditions. Preliminary examination having shown several types of central bodies present, an attempt was made to ascertain whether or not these variations in central body structure could be related to other structural modifications of the mitotic figure. To that end the large metaphase aster in each cell was analyzed with reference to the following points:

1. The central body
   - A. The centrosome
     1. Physical structure (empty, containing more or less scattered materials, or evenly homogeneous)
     2. Size and shape
     3. Degree of demarcation from ray area (demarked distinctly, doubtfully, or not at all)
     4. Stain in contrast to that of ray area (lighter, similar, or darker)
   - B. The centriole
     1. Number
     2. Location in the centrosome
     3. Size
     4. Shape (regular or irregular)
     5. Contour (smooth or rough)
     6. Stain in contrast to that of the centrosome

C. Granules, other than centrioles, occurring near the astral center
1. Number
2. Size
3. Location
4. Similarity to cytoplasmic granules

II. The rays [4]
A. Coarseness (very coarse, medium coarse, delicate, or vague)
B. Shape (rippled or serpentine, undulating or almost straight)
C. Occurrence of small vacuole-like areas among the rays

III. The spindle
A. Size
B. Shape of tip (pointed, rounded, or intermediate)
C. Structure of fibers in contrast to that of astral rays

The various types of centers occurring after Boveri's picro-acetic fixation constitute an unbroken series: the central area may be completely empty; it may contain either small amounts of scattered material, sometimes arranged like the walls of vacuoles, or more abundant material, distributed regularly or irregularly; finally, it may be an evenly-filled, homogeneous region. These centers are about $5 \times 6 \mu$ in size, although there is much variation here.[5] Whether such a series is illustrated by few or many drawings is purely arbitrary. In Chart I three classes are shown: "empty," "scattered," which contain more or less irregularly distributed material, and "even," or homogeneously filled centers, which merge gradually with the ray area and are stained like it.

The various ray configurations also constitute a continuous series: at one extreme they are "rippled" or serpentine; at the other they are "undulating" or almost straight; the intermediate type can best be described as "slightly rippled." The rippled rays have many minute clear spaces, like tiny vacuoles, among their deep curves; the undulating ones have few or none.

Thus when *Chaetopterus* eggs in metaphase are fixed with Boveri's reagent there is a definite relation between central body structure and

[4] It is difficult to select concise terms that describe clearly the differences in the coarseness of rays and in their shape. For example, the use of "very coarse" in contrast to "medium coarse" is somewhat clumsy, but it is necessary to express two degrees of coarseness as contrasted with the condition described by the term "delicate." The terms "rippled" and "undulating" were selected because the former suggests the idea of short sharp curves, in contrast to the long gentle curves suggested by the latter.

[5] Even among cells in metaphase which are alike in the general structure of rays and central bodies there is considerable variation in the size of the center It is practically impossible to secure accurate measurements in most cases, because the central body is demarked from the ray area either vaguely or not at all. In each type, therefore, the cell selected for illustration has a central body of about the average size for its class, based on attempted measurements of about twenty figures of that type. There is also much variation in the shape of the centers: some are round, others quite elongate, others intermediate. The cells selected for illustration as representative for each type have central bodies that are intermediate in shape, being but slightly elongate.

ray structure: all asters with rippled rays have disrupted centers that are either empty or scattered; all those with undulating rays have even centers that are homogeneously filled. There are no exceptions to this relationship. Asters with slightly rippled rays, which are intermediate between these two classes, show all types of centers.

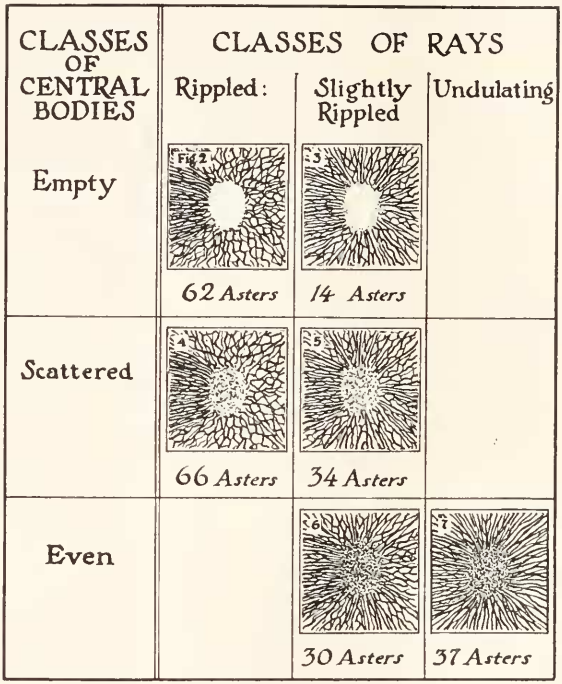


CHART 1. THE RELATION BETWEEN RAY STRUCTURE AND CENTRAL BODY STRUCTURE UNDER OPTIMUM CONDITIONS

Central bodies were studied in metaphase first-cleavage figures in *Chaetopterus* eggs, after using Boveri's picro-acetic fixation, and the standard slide-making procedure. *Result:* Central body structure is related to ray structure: centers are "disrupted", *i.e.*, empty or containing scattered material, if rays are rippled in shape; centers are "even", *i.e.*, homogeneously filled and stained like the ray area, if rays are undulating. Centrioles are not demonstrated.

The slightly rippled rays are obviously intermediate between rippled and undulating rays. Since typically rippled rays are always associated with disrupted centers (empty or scattered) and typically undulating ones with evenly filled centers, the members of this intermediate, or slightly rippled group will hereafter be included with either the rippled or the undulating class, depending upon which group the aster most resembles. Thus the four classes of asters illustrated in Chart 1, in which the centers are empty or scattered and rays either

rippled or slightly rippled (Figs. 2–5) will hereafter be regarded as but minor variations of a single major type, and represented in later charts by an illustration showing a disrupted (scattered) center and rippled rays (Fig. 4). Similarly, the two classes with even centers and rays either slightly rippled or undulating (Figs. 6 and 7) will hereafter be regarded as variations of another major type, represented in later charts by an illustration showing an even center and undulating rays (Fig. 7). These two major types of asters are but two of twelve that occur in this investigation, all of them illustrated in Chart 7 (p. 177). The type with disrupted centers is designated as 1*B* and that with even centers as 2*B*. The basis of classification is discussed on page 176.

Very rarely, *i.e.*, in 16 of the 243 asters studied, one or more granules, like the smaller ones present in the cytoplasm, occur in the even type of center. But since they vary in size, location, and staining capacity, they could not be interpreted as centrioles. These random granules are not the structures Mead illustrated; the kind of central body he described was not produced in this experiment, in which Boveri's picro-acetic reagent was used in the usual way.

Experiment 2. The Effects of Uncontrolled Factors in the Handling of Different Egg-sets Under Optimum Conditions

The purpose of this experiment was to learn whether the percentages of disrupted and even centers (Types 1*B* and 2*B*) occurring in one set of eggs handled under optimum conditions are the same as those occurring in other egg-sets run under supposedly similar conditions but on different days. Table I shows counts of these two types of centers in five different sets, each sample including from 33 to 67 eggs. These data are arranged in the order of increasing percentages of the disrupted type.

When Sets 1 and 2, 2 and 3, 3 and 4, and 4 and 5 are compared, there are only minor differences in the percentages of the two central body types present, and these could be explained by the relatively large errors always involved in reporting small samples. But when Sets 1 and 3, 2 and 4, and 3 and 5 are compared, the differences are large enough to suggest some cause beyond error of sampling. And when Sets 1 and 4, 1 and 5, and 2 and 5 are contrasted it is obvious that the discrepancies are too great to be explained by errors of sampling alone; they must be due either to differences between the living eggs of the various sets prior to fixation, or to uncontrolled modifications of technique. When these relations are analyzed statistically, by determining the value of *P* according to Pierson's method (1924, p. lxx) the ex-

istence of factors which cause differences, other than errors due to small samples, is convincingly demonstrated.[6] The approximate values of P for this material are shown in Table I.

Three problems present themselves: (1) to secure more data concerning the relation between central body structure and ray structure; (2) to explain the cause of the difference in the relative numbers of disrupted and even centers present in different sets after picro-acetic fixation under optimum conditions; and (3) to ascertain what variation of the picro-acetic technique Mead used to demonstrate typical centrioles. The experiments which follow were planned in an attempt to secure information on these points.

Group B. Technique Modified: Slide-Making Procedure Varied; Boveri's Picro-Acetic Fixation

Experiment 3. The Effects of Varying the Depth of Stain (Heidenhain's Hæmatoxylin)

It is conceivable that a center containing considerable irregularly scattered material might, if lightly stained, be listed as belonging to the disrupted (scattered) type, while the same center, if darkly stained, might appear to be evenly filled and would then be counted as an even type. In that event, the differences in the relative numbers of disrupted and even centers in the five sets of the previous experiment might be due to differences in depth of stain.

In that experiment the eggs were stained a deep blue color with Heidenhain's hæmatoxylin. Table II repeats the data of that experiment at the left, and also shows, at the right, the numbers of disrupted and even centers which occurred when eggs of the same five sets were stained a very pale blue. In each set all experimental conditions were identical, in so far as they could be controlled, except the depth of stain.

[6] Pierson's method determines whether a given sample of objects (the 67 eggs of Set 1) having a given number of one class (40 disrupted centers) and a given number of a second class (27 even centers) does or does not belong to the same population as another sample (the 55 eggs of Set 2) with different numbers of the same classes (36 disrupted centers and 19 even centers). These relations are expressed in terms of P. Thus the value of P for Sets 1 and 2, as well as for Sets 2 and 3, is 0.4, meaning that there are 4 chances out of 10 that each pair belongs to the same population, and indicating that the differences between them are not significant. At the other extreme, however, the value of P for Sets 1 and 4 is 0.02, and for Sets 1 and 5 it is 0.001, showing that the chances are, respectively, one out of 50 and one out of 1000, that each pair belongs to the same population. The differences between these sets are therefore significant.

Table I

The effects of uncontrolled factors in the handling of different egg-sets under optimum conditions. Central bodies were studied in metaphase first-cleavage figures in *Chaetopterus* eggs, after using Boveri's picro-acetic fixation, and the standard slide-making procedure. *Result:* Some factor or factors are involved which modify significantly the numbers of the two central body types in the different egg-sets.

Egg-Set Symbol	Number of Cells Studied	Distribution of Astral Types in Each Set		P
		1 B (Disrupted)	2 B (Even)	
1	67	40 60%	27 40%	
				0.4
2	55	36 65%	19 35%	0.1
				0.4
3	48	34 71%	14 29%	0.001
				0.1
4	33	26 79%	7 21%	0.01
				0.1
5	39	35 90%	4 10%	

It is apparent, both by inspection and by the statistical determination of the value of *P*, that the depth of stain with Heidenhain's hæmatoxylin does not modify significantly the percentages of the two central body types occurring in any one egg-set after Boveri's picro-acetic fixation. This is especially obvious when comparing the totals.[7]

[7] This fact does not indicate that various depths of stain would be equally ineffective in modifying the appearance of other central body types, to be described later.

TABLE II

The effects of varying the depth of stain (Heidenhain's hæmatoxylin). Central bodies were studied in metaphase first-cleavage figures in *Chaetopterus* eggs, after using Boveri's picro-acetic fixation. The slide-making procedure was standard except that egg-sets reported at the left in the table were stained darkly and those at the right were stained lightly. *Result:* Differences in depth of stain do not significantly modify the numbers of the two central body types occurring in the different egg-sets.

EGG-SET SYMBOL	EGGS STAINED DARKLY			P	EGGS STAINED LIGHTLY		
	Number of Cells Studied	Distribution of Astral Types in Each Set			Number of Cells Studied	Distribution of Astral Types in Each Set	
		1 B (Disrupted)	2 B (Even)			1 B (Disrupted)	2 B (Even)
1	67	40 60%	27 40%	←0.8→	36	21 58%	15 42%
2	55	36 65%	19 35%	←0.9→	42	27 64%	15 36%
3	48	34 71%	14 29%	←0.6→	44	33 75%	11 25%
4	33	26 79%	7 21%	←0.7→	48	39 81%	9 19%
5	39	35 90%	4 10%	←0.1→	73	61 84%	12 16%
TOTALS	242	171 71%	71 29%		243	181 74%	62 26%

Experiment 4. The Effects of Other Modifications of the Slide-making Procedure

Wide variations as to the speed with which the eggs are passed through the alcohols and xylol, the temperature at which they are embedded, and the thickness at which the ribbon is sectioned, do not change the appearance of the central bodies when Boveri's picro-acetic reagent and Heidenhain's hæmatoxylin are used.

The effects of modifying one other phase of the slide-making procedure were also studied—accidental overheating of the ribbon when it is warmed to bring about its expansion. Different samples of ribbon were variously treated during this operation: in one the ribbon was expanded in the usual manner, remaining opaque without melting throughout the operation; another was so overheated that the ribbon melted

and became transparent for a moment; in a third the same situation was produced but maintained for a longer time; finally, one slide was so extremely overheated that the ribbon not only melted but bubbled and gave off vapors. In the latter case a number of the eggs have a peculiar glassy appearance in the region of the spindle, the inner zone of the astral rays, and the central body area (Fig. 8). This type, produced by faculty technique, is readily recognized and could not be confused with the types occurring under usual conditions.

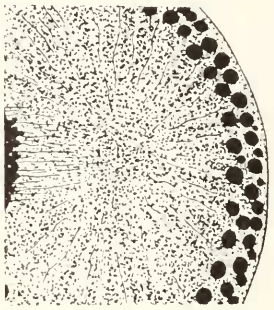


FIG. 8. The glassy type of astral center caused by overheating the ribbon when expanding it.

Central bodies were studied in metaphase first-cleavage figures in *Chaetopterus* eggs, after using Boveri's picro-acetic fixation. The slide-making procedure was standard, except that the ribbon was overheated when expanding it. *Result:* The central body area, the inner part of the ray region, and the spindle have a glassy appearance.

It can therefore be concluded that when *Chaetopterus* eggs are fixed with Boveri's reagent, the inevitable slight variations in the slide-making procedure do not modify central body structure. While this conclusion applies only to the disrupted and even types, it is probable that it also holds true for the other types to be described.

GROUP C. TECHNIQUE MODIFIED: PICRO-ACETIC FIXATION VARIED; REGULAR SLIDE-MAKING PROCEDURE

Experiment 5. The Effects of Varying Simultaneously the Amounts of Picric and Acetic Acids

When eggs are added to the fixative in a vial, even the densest egg suspension includes some sea water, which slightly dilutes the reagent. The present experiment deals with the effects of a series of dilutions, beginning with one part of distilled water and 99 parts of reagent, and ending with 99 parts of water and one part of reagent. To test-tubes

containing 25 cc. of each dilution were added 0.5 cc. of eggs. The amount of sea water included with the eggs was so small, compared with the 25 cc. of diluted fixative, that the effects of any further dilution could safely be ignored. The eggs fixed at 99 per cent, 90 per cent, 75 per cent, 50 per cent, 10 per cent and 1 per cent strength were from one egg-set; those fixed at 30 per cent, 20 per cent, 15 per cent, and 5 per cent were from another egg-set.

Chart 2 shows the percentages of the central body types occurring after fixation with each dilution. When the reagent is from 99 per cent to 50 per cent strength the disrupted and even types previously described (*1B* and *2B*) are the only ones present, their numbers varying at the different dilutions. At 30 per cent strength two new types appear, one having a vaguely delimited centrosome stained darker than the ray area, but without a centriole (*4A*), and one with a similar centrosome but containing one or two centrioles (*5A*). Between 30 per cent and 5 per cent strength these are the major classes. At 5 per cent strength a new type of aster makes its appearance, similar to the one with an even center so frequently described except that it has very delicate rays (*2C*). At 1 per cent strength the asters are very small and vague and their centers are undifferentiated from the ray area. Cytasters are also present,[8] and the chromosomes are abnormal.

The relation between ray structure and central body structure is again obvious, and additional evidence on this point is supplied by the new types. (1) If rays are fixed vaguely, the central area of the aster is entirely undifferentiated from the peripheral part (*2D*). (2) If rays are distinct and rippled the center is disrupted (*1B*). (3) If rays are distinct but undulating, the astral center is filled, its structure varying with the coarseness of the rays, as follows: When rays are either delicate or medium coarse, the center is a homogeneous centrosome, about $5 \times 6\,\mu$ in size, not delimited from the ray area, and stained like it (*2B* and *2C*). When rays are very coarse there are two classes of centers: (*a*) a centrosome, about $4 \times 5\,\mu$ in size, vaguely delimited from the ray area, more darkly stained, and containing no centriole (*4A*); and (*b*) a similar centrosome but with a centriole, either single or double (*5A*). The possible significance of these facts will be discussed after the data of all the experiments have been presented.

The writer reported these results of diluting the reagent to Pro-

[8] As is well known, cytasters are usually produced by methods which bring about artificial parthenogenesis in unfertilized eggs. Occasionally, however, they have been described in normally fertilized eggs. It is possible that some of these were produced by the use of a dilute reagent, as an accidental result of the mode of fixation.

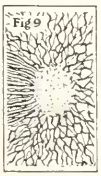
EXPERIMENTAL DATA			DISTRIBUTION OF ASTRAL TYPES AT EACH DILUTION					
			Type 1B	2B	4A	5A	2C	2D
			 Fig 9	 10	 11	 12 13	 14	 15
DILUTION FORMULÆ — Boveri's Picro-acetic Reagent (c.c.)	Distilled Water (c.c.)	NUMBER OF CELLS STUDIED						
99	1	34	32 94%	2 6%				
90	10	34	29 85%	5 15%				
75	25	46	31 67%	15 33%				
50	50	82	80 98%	2 2%				
30	70	39	5 13%	5 13%	12 31%	17 43%		
20	80	52	6 .2%	6 12%	19 36%	21 40%		
15	85	41	1 2%	4 10%	10 25%	25 61%	1 2%	
10	90	47			6 13%	41 87%		
5	95	47			19 40%	17 36%	11 24%	
1	99	40						40 100%

CHART 2. THE EFFECTS OF VARYING SIMULTANEOUSLY THE AMOUNTS OF PICRIC AND ACETIC ACIDS

Central bodies were studied in metaphase first-cleavage figures in *Chaetopterus* eggs, after fixation with Boveri's reagent diluted to various degrees with distilled water. The slide-making procedure was standard. *Results:* (1) Central body structure is related to ray structure, depending upon the coarseness and shape of the latter. (2) Mead (1898) demonstrated centrioles by diluting the reagent. (3) Uncontrolled dilution effects, resulting from the manner in which eggs are added to vials under usual conditions (Exp. 2) are probably responsible for the variations in numbers of the central body types in different egg-sets.

fessor Mead, suggesting that possibly a comparable dilution might have been responsible for the centriole phenomena he found, and hence for the writer's failure to repeat the work with a full-strength reagent. Professor Mead replied that dilution did occur in his experiments, that the results in different preparations were not uniform, and that in many eggs the centers were disrupted. His failure to mention this dilution in his paper is undoubtedly due to the fact that he attached no significance to it—a situation which occurs almost universally in the case of many supposedly minor variations in different steps of the cytological technique. It is worth noting, however, that diluting the reagent is the only modification of the many employed in this investigation which demonstrates centrioles.

Where Mead used sea water to dilute the fixative, distilled water was used in the present work. It is probable that both kinds of dilutions produce similar results, for centrioles can be demonstrated after dilution with either. It is possible, however, that certain differences may exist.

Varied dilution of the reagent explains the occurrence of disrupted and even types of central bodies side by side on the same slide, as well as the variations in their relative numbers in various egg-sets supposedly fixed and run up in the optimum manner (Table I, p. 159). Uncontrolled dilution effects, differing from vial to vial and modifying the central area in asters, are brought about by slight differences in the manner in which eggs happen to be added to the reagent in the vial. If eggs are squirted quickly from the pipette into the vial containing the reagent the mixing is practically instantaneous, but the eggs are handled rather violently. Furthermore, the dish containing the living eggs may become contaminated if the tip of the pipette, which must be held close to the vial when the eggs are added, is splashed with minute droplets of the reagent. For these reasons, eggs are usually allowed to drop gently out of the pipette. Under such conditions the eggs and any sea water accompanying them are more or less segregated near the top of the vial for the first few seconds, the reagent mixing with them from below. It will be shown later (Experiment 9) that fixation occurs within one second; hence the eggs added to the vial are exposed during the first second to various dilutions of the fixative, depending upon whether they happen to be at the top or at the bottom of the mixture of eggs and sea water just added to the reagent.

The data of Chart 2 show that with few exceptions fixation with a full-strength reagent produces disrupted centers (*1B*), and that progressively greater dilutions of the reagent result in filled centers (*2B*, *4A*, and *5A*). In the preceding experiment (Table I) such effects were in-

advertently produced by the manner in which the eggs of the five egg-sets were added to their vials. In Set 1 a considerable percentage of the eggs undoubtedly received a somewhat dilute fixation, since 40 per cent of them have even centers; but those of Set 5 were added in such a manner that only 10 per cent of them were so fixed.

The effect of varied dilutions also explains why, under usual conditions of fixation, about 70 per cent of the centers are disrupted, the remaining ones being even (Table II). Since *Chaetopterus* eggs frequently stick to the bottom of the vial, the writer has heretofore used a narrow vial (9 mm. wide, inside diameter, and 4.5 cm. high), because in it the eggs pile up on top of each other, and few of them touch the bottom. Prior to fixation these vials contained from 4 to 4.5 cc. of reagent; at the time of fixation from 0.5 to 1.0 cc. of eggs were added. The strength of the reagent thus varied from 80 per cent to 90 per cent, the average being about 85 per cent. In the five sets fixed under these usual conditions, 71 per cent of the eggs have disrupted centers and 29 per cent even ones—a result which is comparable to that of the set fixed at 75 per cent strength in the dilution experiment (Chart 2), where the percentages are 67 and 33.

In this dilution experiment the major differences in types and numbers of central bodies occurring at any given strength of the reagent are without doubt primarily the result of the varied dilutions. Within each set, however, the numbers and proportions of central body types were probably also modified by the exact way in which the eggs were added to the reagent. Until the results of this experiment were known the writer had not realized what radical effects can be produced by dilution. Had the eggs been expelled suddenly from the pipette into the fixative instead of being dropped in gently, not only in the present experiment but in the others as well, it is probable that in many cases the numbers of types would have been reduced and their relative percentages somewhat altered.[9]

The fact that dilution of Boveri's picro-acetic reagent causes such marked differences in the structure of rays and central bodies does not mean that this would be true of all cells fixed with any dilute reagent. However, it is probable that the cells of various species are susceptible to dilution effects in various fixatives. For example, although *Echinarachnius* eggs ordinarily show large pleuricorpuscular central bodies

[9] The existence of such an uncontrolled cause of variation in central body types makes it useless to study large egg samples in each group for the sake of reducing the error inherent in small samples. For this reason the number of eggs studied at each variation is seldom more than 50, the average being 36. But although uncontrolled dilution effects may modify the percentages of any type occurring in the eggs of any vial, they do not alter the relation between central body structure and ray structure, which always holds true, without exception.

when fixed with various full strength reagents, including Bouin's, this fluid diluted with 90 parts water produces a minute centriole surrounded by a homogeneous centrosome. The second paper on *Chaetopterus* eggs will demonstrate that the effects of diluting certain chemically diverse reagents are similar to those of picro-acetic dilutions, although the details differ in each case.

Whether dilution effects have played an unsuspected rôle in central body studies of other species remains to be ascertained by future investigations. It may be that in many eggs extensive dilution of the reagent does not modify astral structure. One egg, however, that may be affected in this way is that of *Ascaris* (Fogg, 1931 and many earlier papers); its chorion is so impermeable that it can live in certain strong reagents for hours. When the one usually used, a chloroform-alcohol-acetic mixture, does finally penetrate, it is quite possible that only a very dilute amount of the reagent as a whole, or of one of its components, actually brings about the fixation. This can be determined only if a technique can be developed whereby *Ascaris* eggs can be subjected to instantaneous fixation with full-strength reagents.

Another egg where dilution effects probably occur is that of *Drosophila* (Wilson and Huettner, 1931). It too is impermeable to the reagent. Therefore it is pricked at one end to permit the fixative to enter, and thirty to sixty seconds are required for it to traverse the egg. This may explain the variations found in different mitotic figures in the same stage. In different eggs the variations may be determined by the size of the puncture, which governs the rate at which the reagent enters; but similar effects also occur in different parts of the same egg, probably depending on various dilution effects at different distances from the point of puncture. Here again it would be interesting to compare the results produced by a technique permitting instantaneous, full strength fixation, with those of the present method.

The fact that a certain configuration is produced by a given dilution of a reagent but is not shown when that fixative is used full strength, argues neither for nor against the validity of that coagulation product. Such differences, produced by different dilutions of the same fixative, may have neither more nor less significance than differences produced by chemically diverse reagents. Nevertheless, if certain configurations are produced only by a diluted reagent, that fact must be kept in mind.

The manner in which significant variations are produced in *Chaetopterus* eggs by diluting the reagent suggests that certain precautions should be followed generally in fixing eggs. The amount of reagent should be large enough to prevent any appreciable dilution by the addition of any fluid accompanying the eggs. Furthermore, the eggs should

be added in such a way as to effect instantaneous mixing, in order to prevent inadvertent dilution effects during the first second, when the eggs are fixed.

Experiment 6. The Effects of Varying in Turn the Amounts of Picric and Acetic Acids

In the preceding experiment the amounts of both acids in the reagent were progressively and simultaneously reduced. The present experiment was carried out in order to study the effect on central body structure when each acid in turn is kept constant while the other is varied.

Four egg-sets, run on different days, were used in this experiment. Each illustration in Chart 3 shows the astral configuration resulting from fixation with a different combination of picric and acetic acids. The figures, grouped in four series, are arranged in the order of percentage by weight of both acids present in each case. In series A, shown by the top horizontal row of figures, the picric acid, used as a saturated solution, was kept constant at about 1.2 per cent, and the acetic varied from 0.007 per cent to 20 per cent, the picric here being three times as strong as in Boveri's picro-acetic formula. In Series B, shown by the second horizontal row of figures, the saturated picric acid solution was diluted with two volumes of water (a mixture designated on the chart as "⅓ picric"). This is the same concentration used in Boveri's reagent, which is 0.4 per cent by weight. In Series C, illustrated by the vertical line of figures near the center of the chart, the acetic acid was kept constant at 1 per cent, its concentration in Boveri's formula, while the picric was varied from 0.01 per cent to 1.2 per cent.[10] In Series D, shown by the oblique row of figures in the lower left-hand corner of the chart, both acids were varied, beginning with picric at 0.4 per cent when acetic is at 1.0 per cent, and ending with picric at 0.003 per cent and acetic at 0.01 per cent. This last series, which was reported in the previous experiment, Chart 2, is included here for purposes of comparison with the other three series. Chart 3 also shows the central body type present after fixation with a 1 per cent solution of acetic acid containing no picric acid (Fig. 43), as well as that occurring after fixation with "⅓ picric," containing no acetic acid (Fig. 44).

Table III shows the various formulæ employed. In calculating the

[10] In Chart 3, the picric acid of Series A and B is spoken of as being constant, but this is only relatively true. In Series A, for example, while the acetic acid was varied from 0.007 per cent to 20 per cent, the picric was varied only from 1.2 per cent to 1.1 per cent and thus was relatively constant. The same situation applies to Series B. In Series C the acetic acid actually was kept constant, since 1 cc. of it was added to 99 cc. of different dilutions of picric acid solution.

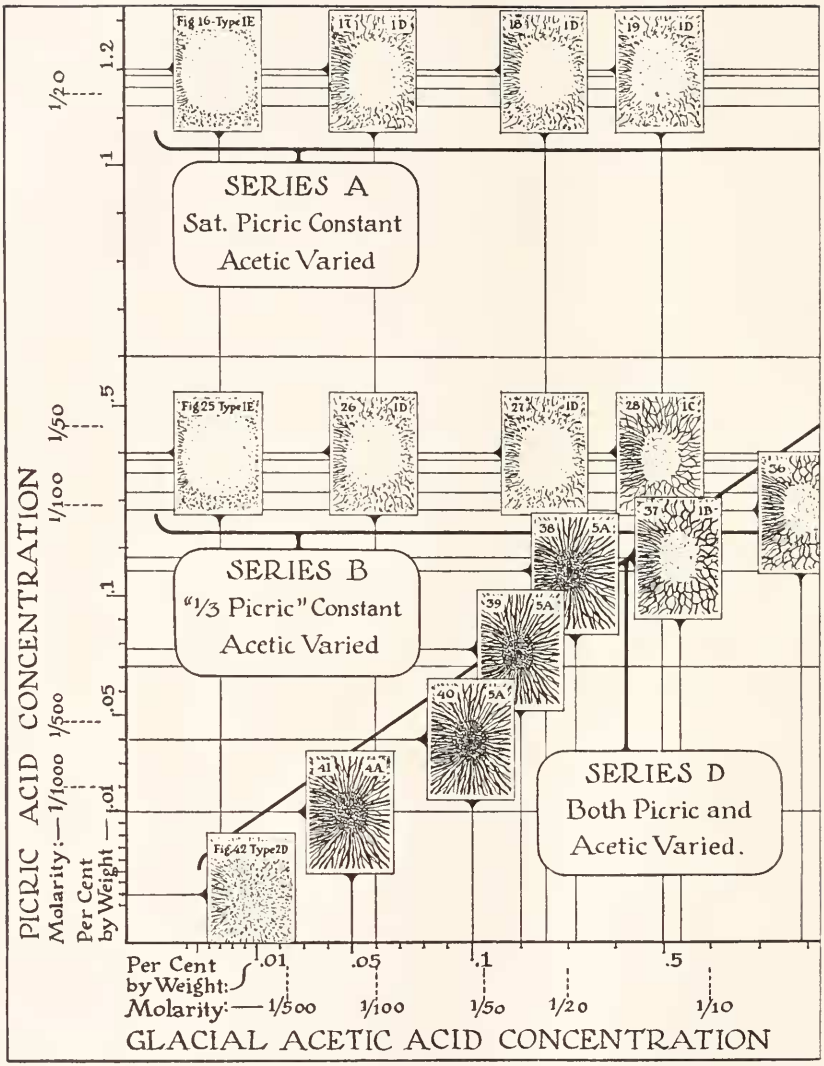
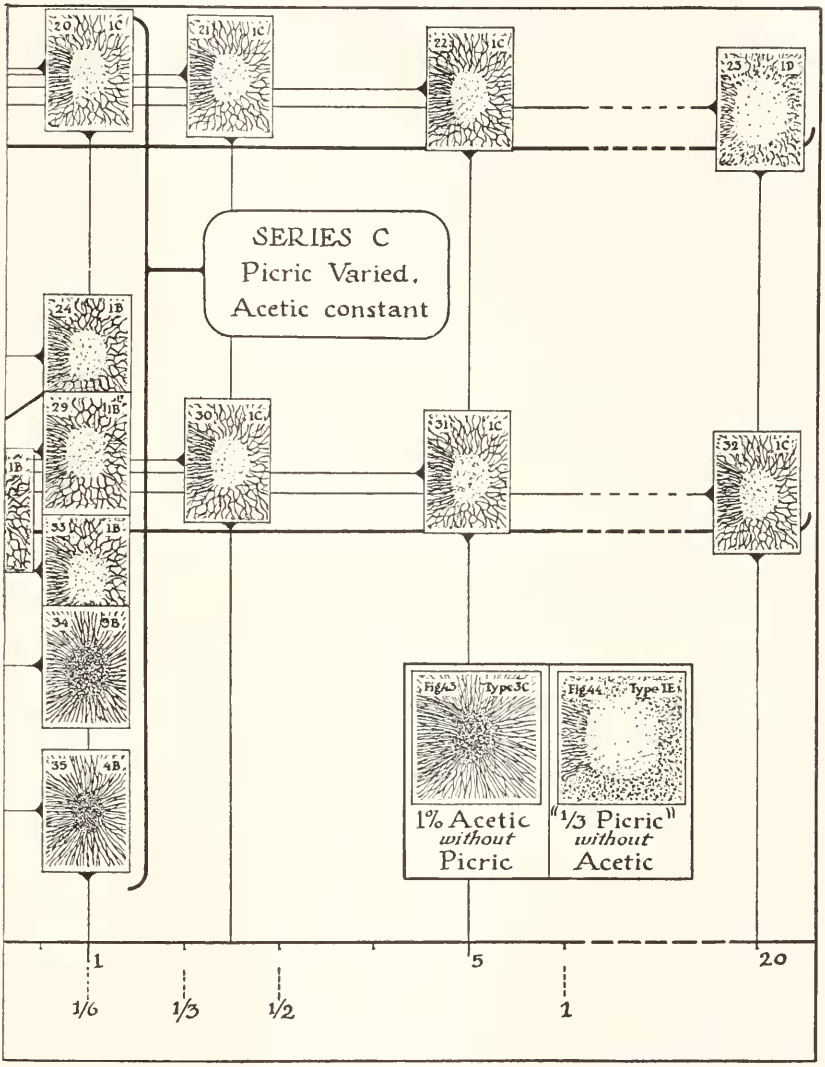


CHART 3. THE EFFECTS OF VARYING IN TURN

Central bodies were studied in metaphase first-cleavage figures in *Chaetopterus* eggs, after fixation with various modifications of the picro-acetic reagent. In some the amount of picric acid was kept constant while the acetic acid was varied (Sets A and B); in others the reverse was done (Set C). The data of the preceding



THE AMOUNTS OF PICRIC AND ACETIC ACIDS

dilution experiment are included for comparison (Set D). The slide-making procedure was standard. *Result:* Only under a very narrow set of conditions are centrioles demonstrated and rays fixed in an undulating and very coarse configuration.

percentage by weight in each formula, 1 cc. of glacial acetic acid was regarded as weighing 1 gram, since its specific gravity is 1.05. The effect upon the solubility of the picric acid (1.22 grams are soluble in 100 cc. of water at 20° C.) caused by changes in room temperature, as well as the possible effect of the presence of acetic acid in the mixture, was ignored. Extreme accuracy in making up the formulæ is meaningless, since slight unknown dilutions were brought about in each case by the addition of small amounts of sea water with the eggs at the time of fixation. The molarities of the two reagents are also shown; they too are only approximately correct.

TABLE III

Formulæ of the picro-acetic reagents used in the experiments reported in Charts 2 and 3

Series A SATURATED PICRIC CONSTANT: ACETIC VARIED			Series B "1/3" PICRIC CONSTANT: ACETIC VARIED			Series C PICRIC VARIED 1% ACETIC CONSTANT					Series D BOTH PICRIC AND ACETIC VARIED		
						Formulæ of Picric Solutions							
Sat. Picric	Acetic	Fig.	Sat. Picric Diluted with 2 Parts Water	Acetic	Fig.	Pts. Sat. Picric	Pts. Water	Picric Sols.	Acetic	Fig.	Picro-Acetic Reagent	Water	Fig.
cc.	*cc.*		*cc.*	*cc.*				*cc.*	*cc.*		*cc.*	*cc.*	
99.99	.007	16	99.99	.007	25	100	0	99	1	20	99	1	29
99.94	.06	17	99.94	.06	26	50	50	99	1	24	75	25	36
99.75	.25	18	99.75	.25	27	33	67	99	1	29	50	50	37
99.5	.5	19	99.5	.5	28	12	88	99	1	33	30	70	38
99.	1.	20	99.	1.	29	6	94	99	1	34	20	80	39
97.5	2.5	21	97.5	2.5	30	1.5	98.5	99	1	35	10	90	40
95.	5.	22	95.	5.	31						5	95	41
80.	20.	23	80.	20.	32						1	99	42

In order to accommodate the drawings, the abscissa of the chart, which shows the percentage of acetic acid, is drawn to various scales in its different parts; *i.e.*, 1 cc. = ½ inch; 0.1 cc. = ¼ inch; 0.01 cc. = ⅛ inch; 0.001 cc. = 1/16 inch. The ordinate showing the percentages of picric acid has been similarly adjusted.

In Series A, B, and C, where there is only slight variation in structure at each modification of the reagent, the illustration on the chart represents the type which is most abundant, based on a study of about 20 eggs in each case. In Series D, where the variation is considerable, large numbers of eggs were studied, but again only the major class occurring after fixation with each dilution is shown, since all of the types occurring are illustrated in Chart 2.

Six new astral types occur in this experiment: (1) one having a disrupted center and delicate rippled rays (*1C*, Figs. 20–22, 28, and 30–32); (2) one with a dense center, stained more darkly than the ray area but not delimited from it, with medium coarse, undulating rays (*3B*, Fig. 34); (3) a similar aster with delicate rays (*3C*, Fig. 43); (4) one with a slightly demarked center, darker than the ray region, with medium coarse, undulating rays (*4B*, Fig. 35); (5) one with a disrupted center accompanied by vague or doubtful rays (*1D*, Figs. 17–19, 23, 26, and 27); and (6) a similar center accompanied by a ray area that is entirely non-radial (*1E*, Figs. 16, 25, and 44).

The results of this experiment are as follows: (1) A typical centriole and its accompanying very coarse undulating rays can be demonstrated only when the percentage by weight of the picric acid is between about 0.01 per cent and 0.2 per cent, and the acetic is at the same time between about 0.05 per cent and 0.4 per cent. Other types of central bodies and ray configurations also occur within this range. (2) If the concentration of the acetic acid is extended to about 2.0 per cent and the picric is held within the range just mentioned, the centers, which are accompanied by medium coarse, undulating rays, are darker than the ray area and are either undemarked from it, *i.e.*, dense (*3B*, Fig. 34), or slightly demarked from it (*4B*, Fig. 35). (3) If the concentration of the picric acid is extended beyond about 0.2 per cent, and that of the acetic beyond about 2.0 per cent, the centers are disrupted and the rays rippled, being either coarse (*1B*), delicate (*1C*), vague (1D), or absent (*1E*), depending upon the formula used. (4) If both picric and acetic acids are very dilute, the picric being less than about 0.005 per cent and the acetic less than about 0.01 per cent, the aster shows only a vague radial organization (*2D*, Fig. 42).

This experiment establishes the fact that the demonstration of a centriole, which is always accompanied by very coarse undulating rays (*5A*), occurs only when the picric and acetic acids are simultaneously within a certain narrow range of concentration.

Experiment 7. The Effects of Varying the Hydrogen Ion Concentration of Boveri's Reagent

Chart 4 shows the effects of the addition of varying amounts of normal sodium hydroxide solution to Boveri's picro-acetic fixative. The pH reported in each case [11] was modified to a slight but unknown degree by the addition of the small amount of sea water which always accompanies the eggs at the time of fixation. In this experiment, however, the egg samples were kept unusually small, so as to change the

[11] The hydrogen ion concentration was determined electrometrically by Mr. Delafield Dubois, who used an apparatus with glass electrodes.

Amounts of normal sodium hydroxide solution listed in Chart 4 were added to 40 cc. of Boveri's reagent.

pH as little as possible. All eggs used in this experiment were from the same egg-set.

The two common central body types previously described occur after using the reagent at its natural pH, 2.2. When the pH is increased to 3.9 the rays are largely suppressed, but the centers remain the same (*1D* and *2D*). When the pH is 4.7, 5.3, or 5.7, rays are undulating and delicate, while the centers are either even (*2C*) or dense (*3C*). In all sets except the one in which the pH was unmodified the mitotic figures are very small, the chromosomes poorly fixed, and the cytoplasm, except in the region of the mitotic figure, crowded with large, darkly-staining granules. A definite relation between ray structure and central body structure is again shown.

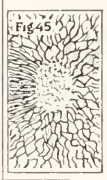
EXPERIMENTAL DATA			DISTRIBUTION OF ASTRAL TYPES AT EACH pH					
			Type 1B	2B	1D	2D	2C	3C
			 Fig 45	 46	 47	 48	 49	 50
pH	c.c. of Normal NaOH added to Boveri's Reagent	Number of cells studied						
2.2	0	20	14	6				
3.9	2	19			15	4		
4.7	4	23					17	6
5.3	6	23					7	13
5.7	7	14					14	

CHART 4. THE EFFECTS OF VARYING THE HYDROGEN ION CONCENTRATION OF BÖVERI'S REAGENT

Central bodies were studied in metaphase first-cleavage figures in *Chaetopterus* eggs, after fixation in Boveri's reagent at various hydrogen ion concentrations. The slide-making procedure was standard. *Result:* Central body structure is related to ray structure; changes occur in both as the pH is varied.

Experiment 8. The Effects of Varying the Temperature of Boveri's Reagent

Eggs from a single egg-set were fixed in two samples of Boveri's picro-acetic fluid, one chilled to 1°C., the other heated to 95°C. These temperatures were of course slightly modified when the eggs and the ever-present sea-water were added, as the latter were at 21° C.

When the reagent is at 1°C., the same two types (1*B* and 2*B*) occur in about the same proportions as when the eggs are fixed under ordinary conditions at room temperature. But at 95°C. all asters have vague rippled rays and disrupted centers (1*D*). In other words, chilling the fixative produces no deviation from the usual condition, but heating it causes changes (Chart 5). The relation between ray structure and central body structure is once more apparent.

Experiment 9. The Effects of Varying the Duration of Fixation with Boveri's Reagent

Eggs were fixed in Boveri's picro-acetic reagent for periods of time varying from one second to six months, as shown in Chart 6. This

EXPERIMENTAL DATA		DISTRIBUTION of ASTRAL TYPES at each TEMPERATURE		
		Type 1B	2B	1D
Temperature of Boveri's Reagent at time of Fixation	Number of cells studied	Fig 31	32	33
1° C	31	19 61%	12 39%	
95° C	30			30 100%

CHART 5. THE EFFECTS OF VARYING THE TEMPERATURE OF BOVERI'S REAGENT

Central bodies were studied in metaphase first-cleavage figures in *Chaetopterus* eggs, after fixation in Boveri's reagent at 1° C. and at 95° C. The slide-making procedure was standard. *Result:* Central body structure is related to ray structure. At low temperatures the central bodies and ray configurations are the same as those occurring at ordinary temperatures; at high temperatures the situation is modified.

experiment shows the speed at which the reagent penetrates and fixes the eggs and also the effects of duration of fixation upon astral structure.

The experimental set-up employed when fixing eggs for periods as brief as one or several seconds was as follows: in order to effect instantaneous mixing, two drops of a thick egg suspension were suddenly squirted into 2 cc. of the reagent, which was kept in motion in a small

Stender dish. This proportion of eggs to reagent weakens the fixative so slightly that any effects of dilution can safely be ignored. After the desired interval of fixation, one or more seconds, the entire mixture was poured into 400 cc. of 70 per cent alcohol, the fluid always used to wash material fixed with a picro-acetic reagent. The addition of 2 cc. of eggs and fixative to 400 cc. of alcohol dilutes the fixative so effectively as to stop its further action. However, to make sure that the alcohol itself, plus the limited amount of reagent added with the eggs, did not modify the coagulation product, a control experiment was run, in which eggs were fixed in 70 per cent alcohol containing 0.5 per cent picro-acetic reagent. The asters in these eggs show only vague rays, and central areas entirely undifferentiated from the peripheral part (*2D*, Fig. 57). Hence the washing in alcohol can be dismissed as a factor in producing the types of astral structure occurring in the experiment. The set-up for fixing eggs for longer periods of time was the usual one.[12]

This experiment shows, first, that complete fixation of the eggs in full strength picro-acetic reagent occurs within one second; (the speed of fixation in dilute reagents is not known). In the second place, it shows that different lengths of exposure to the reagent modify the types of central bodies present. These differences, at the various periods of fixation, are as follows:

Fixation time 1, 3, or 6 seconds: All asters have medium coarse undulating rays; 28 of the 34 centers studied are even (*2B*); the remaining 6 are dense (*3B*).

Fixation time 30 seconds: 35 per cent of the asters have disrupted centers and medium coarse rippled rays (*1B*), and 65 per cent have even centers and medium coarse undulating rays (*2B*). These are the same types which occur after the usual periods of fixation for hours, but the proportions in which they occur are intermediate between those of eggs fixed but a few seconds, when there are no disrupted centers, and those occurring under usual conditions, when there is a high percentage of disrupted centers.

Fixation time 10 minutes or fifteen hours: 90 per cent of the asters have disrupted centers (*1B*), and 10 per cent have even ones (*2B*).

Fixation time thirteen days: only 28 per cent of these eggs are of the disrupted type, 72 per cent having evenly filled centers. The percentages of both types occurring in this set are again intermediate, this

[12] The samples of eggs at the three shortest intervals are intentionally small, because of the effort to keep the egg mass as small as possible. The equally small samples reported for the sets fixed at longer intervals are explained by the fact that this particular batch was fixed a little too early, due to a miscalculation, and but few of the eggs had reached metaphase. However, in spite of the small size of the samples and the error necessarily involved, the major facts are clear.

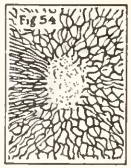
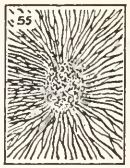
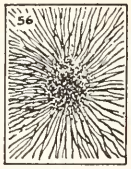
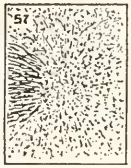
EXPERIMENTAL DATA		DISTRIBUTION OF ASTRAL TYPES AT EACH DURATION of FIXATION			
		Type 1B	2B	3B	2D
Duration of fixation in Boveri's Reagent	Number of Cells studied	 Fig 54	 55	 56	 57
1 second.	*15*		*11* 73%	*4* 27%	
3 seconds.	*10*		*9* 90%	*1* 10%	
6 seconds.	*9*		*8* 89%	*1* 11%	
30 seconds.	*20*	*7* 35%	*13* 65%		
10 minutes.	*20*	*18* 90%	*2* 10%		
15 hours.	*20*	*18* 90%	*2* 10%		
13 days.	*18*	*5* 28%	*13* 72%		
6 months.	*21*		*21* 100%		
CONTROL:- Eggs fixed in 70% Alcohol containing 0.5% Boveri's Reagent.	*20*				*20* 100%

CHART 6. THE EFFECTS OF VARYING THE DURATION OF FIXATION WITH BOVERI'S REAGENT

Central bodies were studied in metaphase first-cleavage figures in *Chaetopterus* eggs, after fixation in Boveri's reagent for various periods of time. The slide-making procedure was standard. *Result:* Central body structure is related to ray structure. Fixation for a few seconds produces only even central bodies and undulating rays; fixation for hours produces in most cases disrupted centers and rippled rays; fixation for months produces the same result as fixation for seconds.

time between those of eggs fixed for hours and those fixed for months.

Fixation time six months: all asters have even centers and undulating rays (*2B*).

In brief, this experiment shows that fixation for a few seconds produces only asters with filled centers, even or dense, and undulating rays; fixation for minutes and hours produces a majority of asters with disrupted centers and rippled rays and a minority with filled centers and undulating rays; and fixation for months returns the aster to the same condition as that produced by fixation for a few seconds. The major result established in the previous experiments is reaffirmed here: ray structure and central body structure are related.

In all of the experiments reported in this paper except this one, the eggs were left in the reagent from six to fifteen hours. This period of fixation falls within the limits of the groups fixed in this experiment at ten minutes and fifteen hours, during which time no modification of central body structure occurred as a result of the length of exposure to the reagent. Hence it can be assumed that they were not affected by differences in length of exposure to the fixative.

IV. Discussion

All of the astral types previously described are arranged in Chart 7, where they are classified on a three-fold basis:

(A) According to differences in structure there are five classes of central bodies:

Number and Name	*Structure*
1. Disrupted.	Not filled. Entirely empty or containing scattered material.
2. Even.	Filled. Not demarked from ray area. Stained like ray area.
3. Dense.	Filled. Not demarked from ray area. Stained darker than ray area.
4. Slightly demarked, without centriole.	Filled. Slightly demarked from ray area. Stained darker than ray area.
5. Slightly demarked, with single or double centriole.	Filled. Slightly demarked from ray area. Stained darker than ray area.

(B) In shape, the rays take two forms: first, rippled or serpentine, and second, undulating or almost straight.[13] Members of the intermediate, or slightly rippled class are included with the other two (p. 156).

(C) On the basis of coarseness there are five classes of rays: (1) very coarse, (2) medium coarse, (3) delicate, (4) vague, and (5) absent.

[13] See footnote 4, p. 155.

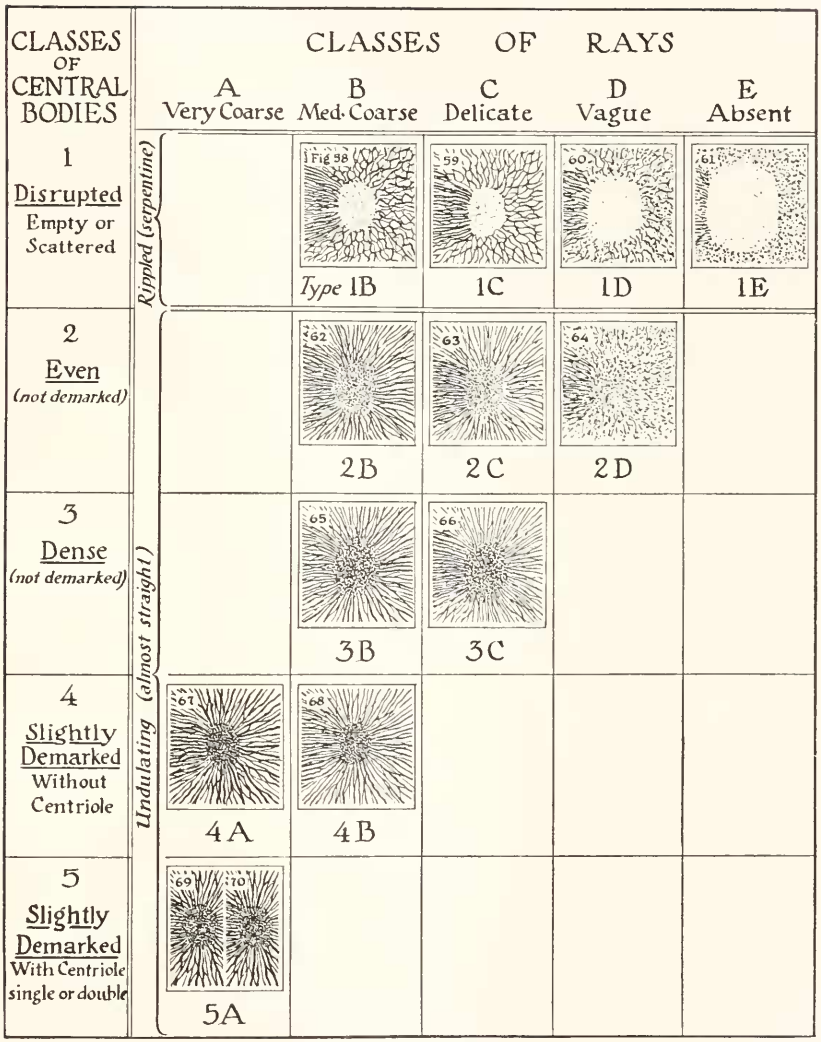


CHART 7. THE ASTRAL TYPES OCCURRING AFTER PICRO-ACETIC FIXATION

Central bodies were studied in metaphase first-cleavage figures in *Chaetopterus* eggs, after fixation with different modifications of a picro-acetic reagent and various changes in the slide-making procedure. *Result:* Central body structure is related to ray structure. If rays are rippled, centers are disrupted; if rays are undulating, but delicate or medium coarse, centers are usually even (or dense) centrosomes, undemarked from the ray area; only if rays are undulating and also very coarse is there a vaguely demarked centrosome containing a centriole, single or double. These occur in 58 per cent of the cases, and they vary in size and shape

The following facts are apparently established by this study, in metaphase first-cleavage figures of *Chactopterus* eggs:

I. The Astral Center is Very Unstable.—The chromosomes maintain their usual form under many modifications of technique, except under a few conditions such as extreme dilution of the reagent, or change in the pH. The spindle, too, is modified only occasionally, and its fibers are affected much less than the astral rays. The aster as a whole, although varying in size, is usually clearly demonstrated. The detailed structure of center and rays, however, shows maximum instability.

II. The Variations in the Structure of the Astral Center are Related to Variations in Ray Structure.—(1) If the ray area is completely nonradial or shows but a vague radial organization, the center is either disrupted (1*D* and 1*E*) or undifferentiated from the ray region (2*D*). (2) If the rays are fixed distinctly and are rippled in shape, the center is never filled; it is always disrupted (empty or scattered), regardless of whether rays are delicate (1*C*) or medium coarse (1*B*). These asters, whose centers are either entirely undifferentiated from the ray area or broken and disrupted, will not be discussed further at this time.

(3) If the rays are fixed distinctly and are undulating in shape, the center is always filled, its detailed structure varying with the coarseness of the rays in several respects: (*a*) Size: the coarser the rays, the smaller is the center. When rays are delicate or medium coarse most centers are about $5 \times 6\,\mu$ in size; when rays are very coarse centers are about $4 \times 5\,\mu$ in size. The latter frequently contain minute centrioles. (*b*) Depth of stain: the coarser the rays, the more darkly does the center stain. When rays are delicate or medium coarse, most centers are even, and stain like the ray area (2*B* and 2*C*); occasionally they are dense, staining somewhat more deeply (3*B*, 3*C*, 4*B*). But when rays are very coarse the center always stains darker than the ray area.[14] (*c*) Demarcation from ray area: the coarser the rays, the greater is the tendency for the center to be demarked from the ray region. When rays are delicate or medium coarse, centers are usually not demarked from the ray area, but gradually merge with it (2*B*, 2*C*, 3*B*, and 3*C*); but when rays are very coarse the center (centrosome) is usually vaguely demarked from the ray region (4*A* and 5A). The degree of demarcation varies not only from center to center, but in

[14] The difference in appearance between the even (2*B* and 2*C*) and the dense (3*B* and 3*C*) types of centers may be due to nothing but slight differences in the depth of stain. This factor, however, does not account for the dark stain of the centers (centrosomes) in asters having very coarse rays (4*A* and 5*A*). These are deeply stained even when the slide is stained very lightly; in fact such slides must be lightly stained, or the centers are clogged with dye.

different parts of the same center; there is no suggestion of a limiting membrane. (*d*) Presence of centriole: only when rays are very coarse does the center show a double zone of differentiation. When rays are delicate or medium coarse, and also in 42 per cent of the asters with very coarse rays, the center is comprised of but a single zone, the centrosome (*2B, 2C, 3B, 3C,* 4*A* and 4*B*). But in 58 per cent of the asters with very coarse rays the center has two zones of differentiation, the centrosome and the centriole (5*A*).

In short, the center behaves more like an area of variable size and form than like a small individualized body of definite size. The coarser and straighter the rays are, the more condensed is the center—*i.e.,* it is smaller, darker, and more delimited. The twelve central body types occurring in the study constitute a graded series, from a completely empty area at one extreme, to a minute centriole surrounded by a vaguely demarked centrosome at the other.

III. When Centrioles are Present They Show Variations in Structure.—Of the 207 asters studied which have very coarse undulating rays and vaguely demarked centrosomes (4*A* and 5*A*: Chart 2, Figs. 11 to 13, representing 187 cases; Chart 3, Fig. 35, representing 20 cases), only 121, or 58 per cent, have centrioles. These have rough and irregular edges and they vary in shape and size. Their configuration in the large aster bears no relation to that of the small one of the same mitotic figure.

IV. The Centriole can be Demonstrated Only under Very Narrow Conditions of Fixation.—To show a centriole when using a picro-acetic mixture, the picric acid must be present in amounts between about 0.01 per cent and 0.2 per cent by weight and the acetic between about 0.05 per cent and 0.4 per cent; all other concentrations used in the present study produce centers without centrioles and rays which are never coarse and undulating. When either or both of the acids are present in very small amounts (Figs. 16, 25, and 42, Chart 3) it is understandable that the reagent may fail properly to coagulate the aster and may thus give meaningless results; but once a concentration is reached which produces very coarse undulating rays and a centriole, it is reasonable to expect that some increase in the amount of either acid would still produce the same kind of coagulation product. Instead of this, however, as soon as the concentration of either exceeds that of the critical zone even slightly, both rays and central bodies are modified. The next paper on *Chaetopterus* eggs will show that equally restricted conditions govern the demonstration of centrioles when diverse reagents are used, both full strength and diluted.

Turning now to the interpretation of these data, it is apparent that the centriole in *Chaetopterus* eggs at metaphase of first cleavage does not behave like a typical centriole, such as is frequently found during the spermatogenesis of many species. The demonstration of the former is dependent upon the simultaneous demonstration of a specific type of ray structure; the latter can be demonstrated whether the mitotic figure is present or absent. The former can be shown only if the fixing agents are present within exceedingly narrow limits of concentration, and even then exhibits variations in size and shape; the latter is readily fixed despite considerable variations in the concentrations of the chemical components of diverse reagents, and shows a similar size and shape in practically all cases. If we are dealing with a true centriole in *Chaetopterus* eggs it is necessary to select one of the twelve centers comprising a continuous series, and regard it as "well fixed," assuming that it is a cell component so constituted chemically that it can be coagulated only under certain narrow conditions, and to dismiss the other eleven types, some of which occur on the same slide, as "poorly fixed."

This behavior of the *Chaetopterus* centriole is not unique. A similar relationship has been established between the structure of central bodies and the structure of rays in cytasters, sperm asters, and first-cleavage figures in *Echinarachnius* eggs (Fry, 1928, etc.) Other studies, soon to be published, demonstrate the existence of a close relation between central body morphology and ray structure in different cell types of various species. Naturally the details differ in each case. In these cells the centrioles exhibit an instability of structure and a relation to ray configuration not generally recognized.

Another and quite different interpretation of the nature of centrioles in *Chaetopterus* eggs is suggested by the data of the present investigation. We may be dealing here, not with an individualized body which exists in the living egg, but with a coagulation product of the focal area of astral rays and spindle fibers. The aster is universally recognized as a complex radial system of converging ray and inter-ray materials, in which the inter-ray substances are crowded out near the center. Since this leaves the central area composed largely or entirely of ray materials, differing both chemically and physically from the peripheral region, is it not possible that this focal area may coagulate differently from the outer parts? Furthermore, its size and physical structure might depend upon the way in which the radial organization of the aster is coagulated —*i.e.*, whether the fixed rays are coarse or delicate, undulating or rippled. Fixation changes in the peripheral portion of the aster may

be related to changes at the center. In such an event, one or more of the configurations produced at the center as coagulation products of the focalized rays and spindle fibers might be small, dark, and delimited, and thus simulate an individualized body. In other words, the demonstration of a small demarked body at the astral center may not necessarily prove the existence of a centriole.

On the basis of this interpretation, the centriole in metaphase first-cleavage figures of *Chaetopterus* eggs is in all probability a focal coagulation artifact. Further consideration of this possibility will be held in abeyance until more data are available in this and other species. In the meantime, in this and future papers, bodies behaving as do *Chaetopterus* centrioles will be spoken of as " focal bodies," to indicate the fact that their structure has a close relation to the detailed structure of focalized rays and fibers, in contrast to that of a typical centriole, which is independent of the exact configuration of rays and fibers.[15]

Some of the confusion in the central body problem may be caused by the possibility that the minute bodies which occur at astral centers actually may belong to several categories, such as the following:

(1) *Random Granules.*—At the astral center of certain cells there are present, after a given fixation, various granules like those in the cytoplasm, which differ in size, number, and location. In *Chaetopterus* eggs they occur very rarely, in the even type of central body (*2B*; discussion p. 157). In *Asterias* eggs they are abundant during the first maturation division after Bouin's fixation. They are likewise abundant in telophase first-cleavage figures of *Cerebratulus* eggs after picro-acetic fixation. This evidence has not yet been reported. Where such granules occur, their configurations in certain asters simulate centrioles, either single or double. Such granules have occasionally been interpreted as centrioles in the past, but it is a simple matter to prove that such configurations are but members of a series.

(2) *Focal Bodies.*—To this group belong the central bodies in *Chaetopterus* eggs described in the present study, central bodies in *Echin-*

[15] The fact that the metaphase centriole of *Chaetopterus* eggs, when present, is either single or double, seems to indicate that it is undergoing division. If such is the case, this is conclusive evidence that we are dealing with a self-perpetuating individualized cell component. But this doubling can also be satisfactorily explained in a way which is in harmony with the suggestion that these structures are focal bodies. However, this question cannot be adequately considered in an investigation which confines itself to metaphase. It will be fully discussed in a later report on first cleavage in *Chaetopterus* eggs, in which centrioles are studied from prophase to telophase, after fixation with reagents which demonstrate them with maximum clearness.

arachnius eggs, and in various other cells to be described in later papers. The morphology of focal bodies is closely related to the exact structure of focalized rays and spindle fibers. In the absence of asters, focal bodies may exist at the spindle-tip, if the latter is sharply focused. If it is blunt, however, as in certain anastral polar-body figures, focal bodies are absent. They are never present before the areas of focalized rays or fibers are formed, and they always vanish when such areas disintegrate, although the outer region of the asters and the middle area of the spindle may still persist. Their behavior differs from that of typical centrioles which exist independently of focalized rays and fibers. Future study may show that certain centrioles which have been assumed to be typical, such as in *Chaetopterus* eggs (Mead, 1898, Wilson, 1930), actually are focal bodies. It remains to be proved, however, whether such focal bodies are individualized structures existing in the living cell, and are unusually difficult to demonstrate, or whether they are merely coagulation artifacts of focalized rays and fibers. In any event their behavior differs so markedly from that of typical centrioles that they should not be confused with them.

(3) *Focal Staining Artifacts.*—These phenomena, described long ago by Fischer (1899) and others, are due to the fact that some asters and spindle-tips, after certain types of fixation, hold the dye with great tenacity at the focal area. In such cases continued destaining makes the body progressively smaller, and finally results in its disappearance. At a certain stage in the process, however, there appears to be a centriole, its size depending upon the degree of destaining. In contrast to such a staining artifact, a focal body exists in the coagulation product as a non-radial body at the astral center, which, if large enough, can be seen in sectioned and unstained material; in other forms, where they are smaller, and when unstained are invisible with white light, they can be demonstrated by ultra-violet photography. A typical staining artifact, on the other hand, contains no demonstrable body at the focal area; instead, the rays or fibers reach the center of the area, and progressive destaining changes the appearance of the center in contour, size, and other points. In the focal body, wide differences in the degree of destaining, short of extreme over- or under-staining, have no effect on its size, since it exists as a non-radial structure at the astral center, and holds the dye somewhat differently than does the surrounding radial area. In some cells the situation is complicated by the fact that both phenomena may be involved: there may be minute focal bodies, in addition to which overstaining may produce staining artifacts in the inner zone of surrounding fibers. These points will be developed in later papers.

(4) *Centrioles.*—These are stable structures which, unlike focal bodies, maintain their characteristic size and shape in spite of extensive modifications in the coarseness and shape of the rays and fibers. In some cells they maintain genetic continuity as individualized bodies from one cell cycle to the next, whether the mitotic figure is present or absent, as in *Drosophila* eggs (Wilson and Huettner, 1931) and in *Amphiuma* leucocytes (Pollister, 1932); in other cases they may disappear during interkinesis, but when present they exhibit a stability of structure unlike that of focal bodies. They are readily demonstrated in their characteristic form by a variety of reagents.

(5) *Blepharoplast-centrioles.*—These are found only in motile cells, or in the forerunners of such cells, with the exception of certain atypical ones, such as the aflagellate sperms of *Ascaris,* recently reexamined by Sturdivant (1931). They have been studied most extensively in the spermatogenesis of animals, where they may arise in the primary spermatocyte or earlier; or they may not appear until the spermatid stage. Like the centrioles of non-motile cells, they are exceedingly stable and show their characteristic morphology when fixed by many different reagents; they are unaffected by changes in the physical structure of focalized rays or fibers; and in some species they are present in the entire absence of such areas. In some cells blepharoplast-centrioles are visible in the living condition. These centrioles are ordinarily thought of as in the same class as those of non-motile cells. This may actually be the case, but the fact that the one is a blepharoplast as well as a centriole, while the other is only a centriole, makes it advisable to give them separate terms which suggest this distinction in their behavior.

(6) *Other Categories.*—There are other types of structures which have been called central bodies such as the following: (*a*) *Bi-lobed bodies of sperm.* In the middle-piece of the echinoderm sperm is a bi-lobed structure of chondriosome material which Boveri misinterpreted as centrioles. That such is not the case was long ago shown by various workers.[16] (*b*) *Erythrocyte centrosomes.* Structures are present during the vegetative phase of erythrocytes in various species which have been interpreted as central bodies. Dawson (1932) has shown that such an interpretation is not valid. (*c*) *Diplosomes of epithelial cells.* Bi-lobed structures lying near the outer part of certain epithelial cells have been identified as central bodies. Whether or not such is actually the case remains to be proved.

Centrioles are generally considered to be the most stable and persistent component of the mitotic figure in the animal cell, and are assumed to play some kind of a formative rôle in connection with the

[16] For a discussion of this see Fry, 1929*a*, p. 105.

origin of asters and spindles, in some cases giving genetic continuity to successive division figures. It is therefore important that we ascertain whether the cell structures we now call centrioles belong to one category or to several, and that we determine the inter-relationships of these classes. Before this can be done, however, the centrioles of many diverse types of cells must be studied, keeping in mind the various modes of behavior, and also the dangers of misinterpreting certain structures that look like typical centrioles but actually may not be so. In the meantime we must be cautious in generalizing from the behavior of one class to that of the others.

V. Summary

In earlier studies of *Echinarachnius* eggs the writer reached the conclusion that their central bodies are the coagulated focal area of converging rays and have no existence as individualized structures in the living cell. The present investigation is the first of a group analyzing central body behavior in a variety of cell types in diverse organisms, for the purpose of ascertaining in each case whether the central body behaves like that of *Echinarachnius* eggs or like a typical centriole, such as that found in many spermatocytes.

The present study confines itself to first-cleavage metaphase asters in *Chaetopterus* eggs, because, in preliminary experiments, the writer failed to find the typical centrioles of that egg previously described by Mead, whose observations were later confirmed by Wilson. The effects upon central body structure produced by the following modifications of technique were observed: the formula, pH, and temperature of the reagent, the duration of fixation, the depth of stain, and other points.

Ray structure is readily modified in coarseness; the shape is also affected, the rays being either rippled (serpentine) or undulating (almost straight). Central body structure is closely related to ray structure: if rays are rippled, centers are disrupted; if rays are undulating but delicate or medium coarse, centers are large homogeneous centrosomes, undemarked from the ray area; only if rays are both undulating and very coarse is there a slightly demarked centrosome, which, in 58 per cent of the cases, contains single or double centrioles, which vary in size and shape.

Centrioles can be demonstrated only under a very limited set of chemical conditions. Among the many modifications of the formula employed, the only ones coagulating centrioles and very coarse undulating rays are those in which the reagent was diluted with about 80

parts of water. In these the percentage by weight of the picric acid is between about 0.01 per cent and 0.2 per cent, and of the acetic acid, between about 0.05 per cent and 0.4 per cent. Any deviations from these conditions produce other types of both centers and rays.

If the central body in *Chaetopterus* eggs is a true centriole, it is atypical in several respects: the great instability of the central body area, the close relation between its morphology and the exact configuration of the fixed rays, and the narrowly limited conditions of fixation under which centrioles can be shown.

The suggestion is made that, as in *Echinarachnius* eggs, the various kinds of central bodies occurring may be various types of coagulation products of the focal area of converging rays, the morphology of the center depending upon the way in which the rays are fixed; only by chance do certain of them simulate individualized structures. Further consideration of this point awaits additional data.

Various classes of minute bodies occurring at the centers of fixed asters are discussed, and the suggestion is made that heretofore we have applied the single term centriole to several very diverse classes of structures, which often look alike but actually behave so differently as to warrant a careful consideration of whether or not they belong to several different categories.

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